Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 152-158

Activation of Mitogen-Activated Protein Kinase by the Human Prostaglandin EP_{3A} Receptor

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Received April 21, 1995

Abstract Mitogen-activated protein (MAP) kinases are involved with cellular proliferation, and while the traditional activators of these kinases have been the growth factor receptors, recent data indicate that G-protein coupled receptors which inhibit adenylyl cyclase can activate MAP kinases as well. We have recently cloned an alternative splice variant of a human receptor for prostaglandin E_2 (PGE_2) which inhibits adenylyl cyclase and as been defined as the EP_{3A} (*Brit. J. Pharmacol.* 112:377, 1994). In the present study the ability of this receptor to activate MAP kinase was examined. In crude lysates of COS-7 cells transfected with the human EP_{3A}, 1 μM PGE_2 stimulated MAP kinase activity ~1.3-fold with an EC_{50} of ~6 nM. Ion exchange chromatography followed by immunoblot analysis showed that the stimulation of MAP kinase activity co-fractionated with immunoreactive MAP-2 kinase (ERK1). This activation of MAP kinase activity by the EP_{3A} receptor may explain the proliferative actions of PGE_2 in some tissues.

Mitogen-activated protein (MAP) kinases have been clearly implicated in the proliferative response of several cell types (1-3). Traditionally, the main signal transduction pathway leading to the activation of MAP kinases was by way of receptors with tyrosine kinase activity such as the epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors. Recently, however, it has been shown that several G-protein coupled receptors which activate G_i proteins can also stimulate $p21^{ras}$ and MAP kinase activity. Examples of these G_i coupled receptors include the α_2-adrenergic (4) , m2 muscarinic (5) and thrombin (6) receptors. With respect to the activation of MAP kinase activity, it is unclear at this time where the convergence begins in the signaling cascades of the growth factor and G_i-coupled receptors. In both cases, however, stimulation of the receptors leads to the phosphorylation of MAP kinases on tyrosine and threonine residues thereby activating the kinases. This is believed to be followed by a translocation of the kinase

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0006-291X/95 $5.00

to the nucleus and the phosphorylation of transcription factors leading to cellular proliferation.

It is known that prostaglandin E_2 (PGE_2) can affect cell growth and recently it has been shown to stimulate mitosis in fetal rat bone cell cultures (1). Interestingly, this effect appeared to be independent of changes in cAMP levels suggesting a novel signaling mechanism. Four subtypes of receptors for PGE_2 have been defined which are known as EP_1, EP_2, EP_3 and EP_4 (7). The EP_2 and EP_4 subtypes are known to stimulate cAMP formation while the EP_1 and EP_3 subtypes stimulate intracellular Ca^{2+} mobilization and inhibit cAMP formation, respectively. We have recently cloned an alternative mRNA splice variant of the human EP_3 receptor (8) and have shown that it inhibits cAMP-dependent reporter gene expression in a pertussis toxin sensitive manner. To examine the possibility that this receptor (EP_{3A}) also couples to a MAP kinase pathway, we examined the potential of the EP_{3A} to stimulate MAP kinase activity in transiently transfected COS-7 cells. We have found that PGE_2 stimulates this activity in a dose dependent manner and that it associated with the MAP-2 isoform of the MAP kinases.

METHODS

COS-7 Cell Transfection. COS-7 cells were transiently transfected with pBC/EP_{3A} which is a eukaryotic expression plasmid encoding the human EP_{3A} prostaglandin receptor (8). Transfection was by DEAE-dextran/DMSO shock after which the cells were incubated for 48-72 hrs in DMEM containing penicillin/streptomycin and 5% fetal bovine serum (Hyclone). In some experiments COS-7 monolayers were trypsinized 24 hours after transfection and cells transferred to 6 well tissue culture plates (Falcon).

MAP Kinase Assay. Transfected COS-7 cells in DMEM were serum-starved overnight and were stimulated with PGE_2 (Cayman Chemical) or phorbol myristate acetate (PMA, Sigma) and incubated at 37°C in DMEM. Preparation of COS-7 cell lysates and the MAP kinase assay were performed essentially as described (9). Cell monolayers were washed with ice cold phosphate buffered saline (PBS) and were scraped into ice cold lysis buffer (50 mM ß-glycerophosphate, 1 mM EGTA, 2 mM $MgCl_2$, 100 µM sodium vanadate, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF, 20 µM pepstatin, 20 µM leupeptin, pH=7.2). Cell debris was sedimented at 12000 X g for 15 min and 10 µL of the supernatant was utilized for the MAP kinase assay using myelin basic protein (MBP, Gibco/BRL) as the substrate. Assays contained 1 mg/mL MBP, 12.5 mM $MgCl_2$, 25 µg/mL protein kinase A inhibitor (PKI, Sigma), 50 µM [^{32}P]γ-ATP (2200 cpm/pmole, NEN) in a final volume of 40 µL. After 15 min at 37°C reactions were terminated by the addition of 10 µL of 25% (w/v) trichloroacetic acid. Aliquots (25 µL) were spotted onto P-81 phosphocellulose filter paper (Whatman) and were washed (4 x 5 min) with 75 mM phosphoric acid followed by a 2 min acetone wash. Filters were counted in Safety-Solve (Research Products International) and the results normalized to the protein concentration of the cleared lysates. Background binding to filters was determined in the absence of cell lysate.

Q-Sepharose Chromatography. In some experiments cell lysates were fractionated over Q-sepharose (Pharmacia/LKB Biotechnology). Transfected COS-7 cells in 15 cm tissue culture dishes were incubated either in the presence or absence of 1 µM PGE_2 for 5 min at 37°C and were scraped into 2-3 mL of ice cold lysis buffer. After centrifugation

(12000 X g) for 15 min at 4°C, lysates were matched for protein concentration and diluted ~5 fold in column buffer (50 mM ß-glycerophosphate, 1 mM EGTA, 2 mM $MgCl_2$, 100 µM sodium vanadate, 1 mM dithiothreitol, 1 mM PMSF, pH=7.2) and were loaded onto a 4 mL column of Q-sepharose at 4°C. The column was washed with 2 bed volumes of column buffer and was eluted with a linear gradient of 0→500 mM NaCl in column buffer. Fractions were collected (1 mL) and were used for western blot analysis and determination of MAP kinase activity. Protein concentrations were determined using Coomassie Plus Protein (Pierce) according to the manufacturer's instructions.

Western Blot Analysis. Aliquots (15 µL) from the Q-sepharose chromatography were electrophoresed on 12% denaturing SDS polyacrylamide gels using a mini-Protean II gel apparatus (BioRad) and were transferred to nitrocellulose at 100 V for 1 hr using a mini-trans blot cell in 192 mM glycine/25 mM Tris, pH 8.3. The blots were probed with a primary mouse monoclonal antibody to MAP-2 kinase (ERK1, Gibco/BRL) and with a secondary anti-mouse IgG coupled to horse radish peroxidase (Amersham). Both antibodies were used at 1 to 1000 dilutions and detection was done using enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Data Analysis. The kinetics of PGE_2-stimulated MAP kinase activation were analyzed by ANOVA for statistical significance. Significance between treatment groups was determined with the Newman-Keuls' multiple range test. The EC_{50} for PGE_2 stimulation was determined by nonlinear regression analysis using Prism (Graphpad). All error bars represent the standard errors of the mean.

RESULTS

COS-7 cells were transiently transfected with plasmid DNA encoding the human EP_{3A} prostaglandin receptor and a time course for the stimulation of MAP kinase activity by PGE_2 was determined. The results of this study are shown in Figure 1 in which MAP kinase activity was measured in crude cell lysates following incubation of the cells with either 1 µM PGE_2 or 100 nM phorbol ester (PMA). At the earliest time point (2 min) PGE_2 produced a 1.2 fold stimulation of MAP kinase activity which was essentially equal to the induction of MAP kinase activity caused by a maximally stimulating concentration of PMA. The stimulation of MAP kinase activity by PGE_2 decreased with longer incubation times and was close to baseline after 15 min. EGTA and an inhibitor of protein kinase A (PKA) were included in the assays to inhibit the phosphorylation of the substrate (myelin basic protein) by protein kinase C and PKA, respectively.

In Figure 2, the dose-response relationship for the stimulation of MAP kinase activity by PGE_2 was examined in COS-7 cells transfected with the EP_{3A}. Again, in crude cell lysates, PGE_2 was able to maximally stimulate MAP kinase activity 1.3 fold with an EC_{50} of ~6 nM. This is similar to the EC_{50} of PGE_2 (15 nM) for the inhibition of specific [^{3}H]PGE_2 binding to membranes prepared from EP_{3A}-transfected COS-7 cells (8). Over the same range of concentrations, PGE_2 did not stimulate MAP kinase activity in untransfected COS-7 cells (data not shown).

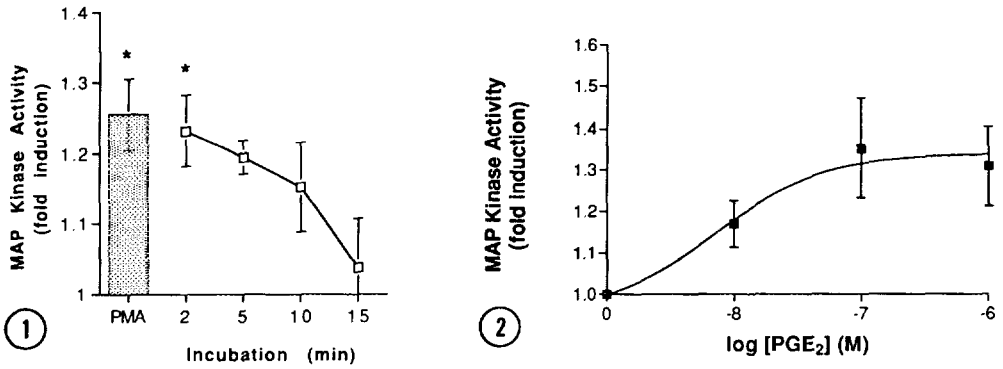

Figure 1. Time course for the activation of MAP kinase by 1 μM PGE_2 in COS-7 cells transfected with the human EP_{3A} receptor. Transfections and MAP kinase assays were done as described in Methods. Data are expressed as the fold induction of MAP kinase by PGE_2 over the unstimulated controls. Time points for each experiment were done in duplicate and the data shown above are the average of 4 independent experiments. Asterisk (*) indicates statistical significance at $p < 0.05$. PMA was used at a final concentration of 100 nM for 10 min.

Figure 2. Concentration/response for the activation of MAP kinase by PGE_2 in COS-7 cells transfected with the human EP_{3A} receptor. Drug incubations were for 5 min and MAP kinase assays were done as described in Methods. Data are expressed as the fold induction of MAP kinase by PGE_2 over the unstimulated controls. Each point was done in duplicate and the data shown above are the average of 3 independent experiments. PMA controls done at the same time yielded a 1.5 ± 0.1 fold induction over the unstimulated controls.

To determine the nature of the kinase involved in the stimulation of MAP kinase activity by PGE_2, cell lysates were fractionated by ion exchange chromatography over Q-sepharose and MAP kinase activity and immunoreactive MAP-2 kinase were measured. The results of one such experiment are shown in Figure 3 in which EP_{3A} transfected COS-7 cells were incubated in the absence (control) or the presence of 1 μM PGE_2. In both cases, peak activities of MAP kinase activity (upper panel) and immunoreactive MAP-2 kinase (lower panel) were present in fraction 11; however, for cells incubated with PGE_2, there was a 2 fold greater activity of MAP kinase as compared with control. In contrast, the amount of immunoreactive MAP-2 kinase was the same for both the PGE_2 incubated cells and the controls, indicating that the greater activity of MAP kinase observed for the PGE_2 incubated cells was not simply due to a greater amount of MAP-2 kinase.

DISCUSSION

Previous pharmacological studies and more recent molecular biological studies have shown that EP_3 prostaglandin receptors inhibit the activity of adenylyl cyclase in cells and tissues (7). This inhibition of adenylyl cyclase and cAMP formation occurs through an interaction with a G_i protein which can be blocked by

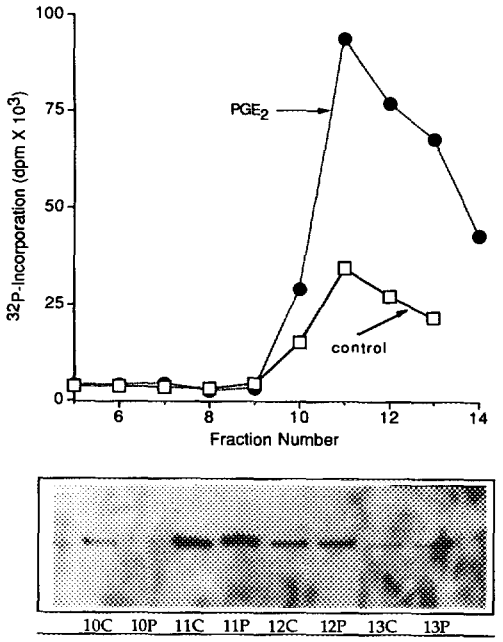

Figure 3. MAP kinase activity (upper panel) and immunoreactive MAP-2 kinase (lower panel) following Q-sepharose chromatography of COS-7 cells transfected with the human EP_{3A} receptor and stimulated with 1 μM PGE_2. Control represents the results obtained from unstimulated cells which were run in parallel. Columns were loaded with equivalent amounts of protein. Transfections, MAP kinase assays and western blotting were done as described in Methods. Data are from a representative experiment which has been repeated 3 times. In the lower panel C represents fractions from the control and P represents fractions from PGE_2 stimulated cells.

prior treatment of the cells with pertussis toxin. Other receptors, such as the m2 muscarinic, thrombin and α_2-adrenergic also inhibit adenylyl cyclase in a similar fashion and have recently been shown to activate MAP kinase activity as well. In the present study, the potential of EP_3 receptors to activate this novel pathway was examined in COS-7 cells transfected with the cloned human EP_{3A} receptor.

As shown both in crude cell lysates and in partially purified preparations of MAP kinase, PGE_2 was able to stimulate the activity of MAP kinase in EP_{3A} transfected COS-7 cells to the same extent of that obtained with PMA, a known activator of this pathway. The time course of the activation of MAP kinase activity by the EP_{3A} was comparable that obtained with COS-7 cells transfected with the m2 muscarinic and α_2-adrenergic receptors (10), suggesting a similar mechanism of activation. In the case of the m2 and α_2 receptors, the activation of MAP kinase was found to occur through the generation of free $\beta\gamma$ subunits from G_i and to involve the activation of $p21^{ras}$. In the present studies, the activation of MAP kinase activity by 1 μM PGE_2 in EP_{3A} transfected COS-7 cells was completely blocked by preincubation with pertussis toxin indicating the participation of G_i in this pathway (data not shown).

The MAP kinase assay utilized in this study measures the phosphorylation of myelin basic protein (MBP). As other kinases (eg. PKA and PKC) can also phosphorylate MBP (11) we used ion exchange chromatography over Q-sepharose to fractionate cell lysates and found that PGE_2 stimulated MAP kinase activity co-eluted with immunoreactive MAP-2 kinase. In agreement with others (12), there was a greater fold activation of MAP kinase activity in column fractions as opposed to crude cell lysates. This is likely due to the purification of MAP kinase away from PKA or other regulatory proteins during column chromatography. PKA is known to downregulate MAP kinase activity (13) and omission of the PKA inhibitor from our assay buffer reduced MAP kinase activation.

A significant development in the molecular characterization of the EP_3 receptors has been the discovery of numerous alternative mRNA splicing variants of these receptors (8, 14-16). With respect to the human species alone there are at least six alternative splice variants which have the same amino acid sequences in their amino termini and transmembrane domains but differ in their carboxy termini. It appears that these differences impact the desensitization of these receptors and their coupling to either the adenylyl cyclase or calcium dependent second messenger pathways. The present finding that EP_{3A} receptors can also activate MAP kinase activity will be important towards understanding the functional differences that may exist between the EP_3 receptor variants.

Acknowledgments. The authors thank Allergan,Inc. and the HLB Training Grant (HL07249) for the support of this work. Additional support has also been provided to J.W.R. by the National Eye Institute (EY09355), the Arizona Affiliate of the American Heart Association, the Glaucoma Foundation and the R.W. Johnson Pharmaceutical Research Institute.

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